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14. ABSTRACT Pulsed power for biological investigations newly developed at USC include a fast diode-based systems designed to drive cell suspensions in a microscope slide electrode microchamber for observations of living cells during pulse exposure with pulse durations from 3 ns to 30 ns and electric fields from 1 MV/m to 10 MV/m. Nanoelectropulse responses have been observed in vitro with the following cell lines (human unless otherwise noted; ATCC catalog number in parentheses): Jurkat T lymphoblasts (TIB-152), RPMI 8226 multiple myeloma cells (CCL-155), SKOV-3 ovarian cancer (HTB-77), AsPc1 pancreatic cancer cells (CRL-1682), U-87 MG glioblastoma cells (HTB-14), MCF-7 breast adenocarcinoma (HTB-22), WI-38 fetal lung fibroblasts (CCL-75), WI-38 VA-13 sub-line 2RA (SV40-transformed WI-38; CCL-75.1), C6 rat glioma cells (CCL-107), NIH 3T3 murine fibroblasts (CRL-1658), normal T cells (from healthy donors), and bovine adrenal chromaffin cells and rabbit cardiomyocytes (both primary cultures). AsPc1 pancreatic cancer cells were also implanted into athymic nude mice for evaluation of solid tumor responses in vivo.					
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“Pulsed Electric Fields for Biological Weapons Defense”

AFOSR Grant No. FA9550-04-1-0107

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OBJECTIVES

The overall objectives of the program are to conduct electroporation experiments using a range of electrical pulse parameters to

- Determine the response of cells to the fields,
- Determine effectiveness of responses to tailored electromagnetic fields.
- Develop technology for application of pulses to small enclosed containers and envelopes
- Determine effect of the application of ultra-short pulses on intracellular gene expression leading to toxicity for spores and bacteria, and on the integrity of cytoplasmic and internal membranes and other intracellular structures
- Determine whether there will be device applications incorporating the cell response to the fields, that can form the basis for simply implemented methods of decontamination.

STATUS OF EFFORT

Background:

Biological responses to nanoelectropulses. Nanosecond, megavolt-per-meter pulsed electric fields nondestructively perturb the intracellular environment, causing calcium bursts [1, 2], eosinophil sparklers [3], vacuole permeabilization [4], nuclear chromatin rearrangement [5], activation of excitable cells (cardiac myocytes and adrenal chromaffin cells) [6], and the appearance of apoptotic indicators such as release of cytochrome c into the cytoplasm [7], loss of mitochondrial membrane potential, and caspase activation [8, 9]. Nanoelectropulse-induced killing of cancer cells and shrinking of tumors has been demonstrated in vitro and in vivo [10,11].

In addition to these responses in the cell interior, nanoelectropulse exposure also induces phosphatidylserine (PS) externalization — translocation of PS from the cytoplasmic face of the plasma membrane to the cell exterior — a normal event in platelet activation and blood coagulation [12], a diagnostic feature of apoptotic cells which serves as a physiological semaphore for their phagocytic removal [13], and a means of intramembrane signal transduction in lymphocytes [14]. The ability to activate this signal remotely, with non-ionizing, non-thermal (high power, but low total energy), non-invasive electric pulses may be useful in both research and clinical settings.

All of these effects are mediated by the generation of perturbative potentials on cellular structures for periods shorter than the charging time constant of the plasma membrane (tens to hundreds of nanoseconds for mammalian cells of various sizes and shapes) [3,15]. Although the electrical power associated with these pulse exposures is high (megawatts), nanosecond pulse durations limit the total energy delivered to nanojoules

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per cell, and there is no immediately apparent physical damage at the cellular level. Despite these low energies, the initial effects are abrupt, well-defined, and apparent using fluorescence microscopy within milliseconds.

Dose response and pulse parameters. The unique electrophysical properties of the biological cell, which can be modeled in its simplest form as a dielectric shell enclosing and suspended in conductive media and containing within its bounded volume smaller dielectric shells (membrane-bound intracellular structures), provide a basis for understanding these responses [7,9,16], but more extensive experimental studies are needed to refine the models, to systematically define the sensitivities of different cell types, and to identify and characterize the mechanisms underlying the response patterns that have already been observed.

It is of particular importance to chart the minima of the pulse parameters — electric field, pulse rise time and duration, pulse count and repetition rate — required to produce these biological responses. Large pulse doses are likely to produce multiple effects, which may be useful for cancer therapy or other applications where cell killing is the overriding desired outcome, but in order to draw out and delineate the molecular disequilibria caused by nanoelectropulse exposure it will be necessary to minimize the perturbations to the cellular machinery instead of delivering an overwhelming dose.

Previous studies indicate that nanosecond pulse effects may be absent when the electric field amplitude is less than about 1 MV/m [17], but a careful review of these observations is essential in order to confirm this threshold for the various cellular responses mentioned above, and to investigate whether the failure to detect a response in each case is a consequence of a true threshold or whether it results from the relative insensitivity of the assay or the instrumentation. Other variables may affect the level of the observed threshold (for example, electroporation with 4 ns pulses is observed only at high pulse repetition rates [18], and the effects of different combinations of pulse parameters on the final response is undoubtedly complex.

Nanoscale pulse generator engineering. A critical obstacle standing in the way of the development of experimental programs in this area is the difficulty of constructing and maintaining pulse generators with the requisite capabilities. For in vitro biological loads in cuvettes or electrode microchambers, or for exposure of tissue in situ using needle or parallel plate electrodes, the pulse generator output must typically provide tens or hundreds of amperes at hundreds or thousands of volts with rise and fall times on the order of a nanosecond or less, and the pulse must be delivered to the load without degradation by reflections or differential frequency effects. That is a tall order. Observations of cells exposed to pulses as short as 3 ns have been carried out in controlled impedance fabricated chambers [18], but only with custom pulse generator engineering and metrology. Extending these studies to shorter, higher field pulses, and to biological systems in the uncontrolled environments of living tissue, and enabling biologists to conduct experiments without the assistance of electrical engineers will require new approaches to pulse generation instrumentation.

One way to reduce the high-voltage, high-current demands of megavolt-per-meter nanosecond pulse generation is to limit the volume in which the field is produced. High aspect ratio field enhancement at the tip of a charged point or projection is a well-known

phenomenon, and it may be possible to take advantage of this effect by forming electrodes with arrays of carbon nanotubes (CNTs), bio-compatible metal nanowires, or other high-aspect ratio nanoscale structures for delivery of nanoelectropulses to biological systems. For example, following the analysis of Kokkorakis et al. [19], a 5 volt potential on a single CNT can produce electric fields of 20 MV/m or higher over distances within a few nanometers of the tip of the tube, depending on the system geometry and the dielectric environment. Not only does this simplify the pulse generator engineering problem, but the nanometer dimensional scale of these novel electrodes also makes possible the very highly localized application of these new stimuli so that we may envision pulse exposures involving only small regions of the cell — individual organelles or nanometer-size compartments in the cytoplasm or isolated domains of the cell membrane.

Even with nanoelectrodes, delivery of nanosecond electric pulses with sharp rising and falling edges requires careful engineering of the transmission of the pulses from the pulse generating circuit to the terminating electrodes. This can be greatly simplified if the pulse generator output and the load are very close together. A nanoelectrode design which produces megavolt-per-meter fields with potentials of only a few volts enables the consideration of an integrated nanoelectropulse generator, in which CMOS microelectronic circuits drive nanoelectrode arrays assembled on or bonded to the integrated circuit metallization. By eliminating connectors and cables between the pulse generator and the load, inductances and capacitances are minimized and much more easily managed. A nanoelectropulse-generator-on-a-chip constructed along these lines could be combined with a microfluidic network for efficient observations of single cells or groups of cells, and could also become a component of a minimally invasive system for delivering nanoelectropulses to tissues in situ (Figure 1).

Numerous methods for probing and manipulating cellular components at subcellular resolution have been reported, including micro-electrode electroporation [20,21] and microfabricated intracellular optical sensors [22], and CNTs are a current research focus as potential vehicles for delivery of materials into cells [23]. Although nanotube-based field emission arrays have been studied intensively in the past few years, the use of CNTs and other nanostructures in fixed arrays as electrical (or mechanical) probes in biological systems has not received much attention. Investigators at Oak Ridge National Laboratory have developed a method for conjugating DNA sequences to CNTs and presenting this genetic information to the intracellular environment, where it is transcribed and expressed [24,25]. Several groups are working on bioelectrical interfaces incorporating microelectronics [26,27,28], but with conventional electrode materials.

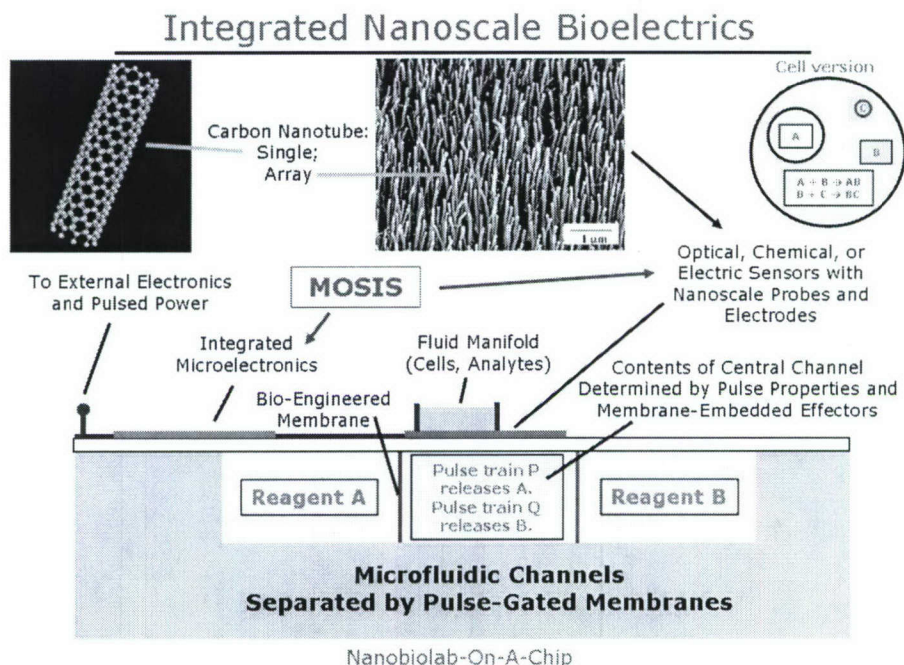


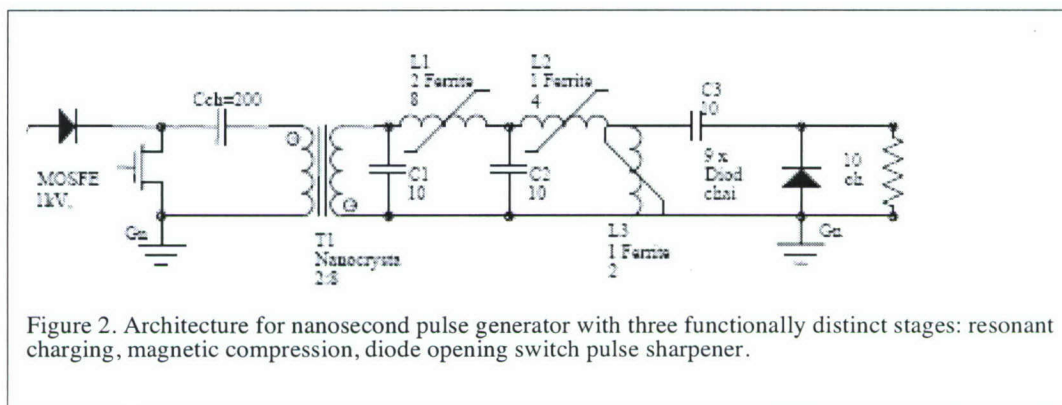
Figure 1. Nanobiolab-on-a-chip with CMOS microcircuit drivers and sensors, nanotube electrical interface to cells, and microfluidic control of reagents and cell suspensions.

Physical and electrical models of bionanosystems. Direct observation of nanosecond events in living biological systems is challenging and for the most part beyond the practical reach of instrumentation and methods available today. A notable exception to this is the effort at Old Dominion University to monitor membrane potential changes in real time during nanoelectropulse exposure using potential-sensitive fluorescent dyes [29]. Molecular dynamics (MD) and micro- and macroscale modeling with field solvers and electrical circuit representations can be used profitably to create simulations for consistency checks and verification of proposed nanoelectropulse perturbation mechanisms, and to generate hypotheses for testing with available laboratory resources. In order to identify the molecular mechanisms operative on a nanosecond time scale during electroporation of phospholipid bilayers, for example, MD simulations of phospholipid bilayers in high electric fields can suggest details of electroporation kinetics and dynamics not directly accessible by experiment [30,31].

Continuum [32] and transport lattice circuit models [33] based on empirical membrane properties and stochastic interpretations of the measured conductance and capacitance of bilayers in electric fields led to predictions by Weaver [34] that nanosecond, megavolt-per-meter pulses cause the formation of numerous, nanometer-diameter pores in all cell

membranes — external and internal — in a system, unlike longer, low-field pulses, which produce the fewer, of larger pores associated with conventional electroporation. Persistent experimental efforts to demonstrate this "supra-electroporation", initially yielding only negative results, finally succeeded, with appropriate fluorescent dyes and high electric fields at high pulse repetition rates, in detecting the nanoelectropulse-driven electropermeabilization of cell membranes [18].

Pulse generator development. For biological applications, including those in the clinic and the field, pulse generators and their associated delivery systems must be compact, robust, and capable of driving kilovolts with nanosecond and sub-nanosecond rise times across low impedance loads consisting of conductive suspensions of electrolytes, soluble macromolecules, and minimally soluble or insoluble matrices of protein, carbohydrate, and lipid. Ideally they will provide the operator with considerable flexibility in pulse amplitude, duration, and repetition rate. Pulse generators for biological investigations developed at USC include MOSFET- and fast recovery diode-based systems designed to drive cell suspensions in a microscope slide electrode microchamber for observations of living cells during pulse exposure with pulse durations from 3 ns to 30 ns and electric fields from 1 MV/m to 10 MV/m [37,38,39]. A different, more recently implemented architecture for both aeronautical combustion and biological applications uses resonant charging, magnetic compression, and a diode opening switch for pulse sharpening (Figure 2).



Cells and biological systems. Nanoelectropulse exposure responses have been observed in vitro with the following cell lines (human unless otherwise noted; ATCC catalog number in parentheses): Jurkat T lymphoblasts (TIB-152), RPMI 8226 multiple myeloma cells (CCL-155), SKOV-3 ovarian cancer (HTB-77), AsPc1 pancreatic cancer cells (CRL-1682), U-87 MG glioblastoma cells (HTB-14), MCF-7 breast adenocarcinoma (HTB-22), WI-38 fetal lung fibroblasts (CCL-75), WI-38 VA-13 sub-line 2RA (SV40-transformed WI-38; CCL-75.1), C6 rat glioma cells (CCL-107), NIH 3T3 murine fibroblasts (CRL-1658), normal T cells (from healthy donors), and bovine adrenal chromaffin cells and rabbit cardiomyocytes (both primary cultures). AsPc1 pancreatic cancer cells were also implanted into athymic nude mice for evaluation of solid tumor responses in vivo. In addition the following microorganisms were studied: *Bacillus*

atrophaeus and isolates from dental patients including *Staphylococcus*, α -hemolytic *Streptococcus*, *Actinomyces*, and *Candida* species.

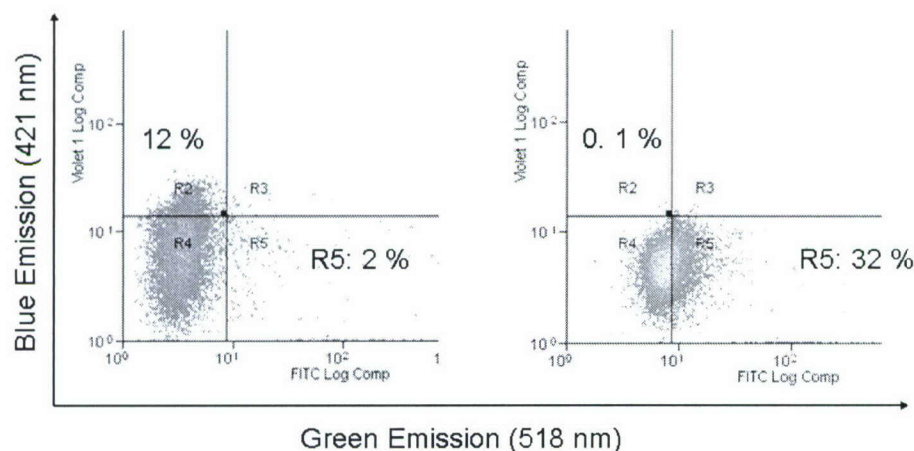


Figure 3. Flow cytometric analysis of spectral red shift (increased ratio of green/blue emission) of Hoechst 33342 fluorescence in pulsed RPMI 8226 human multiple myeloma cells. Regions R2 and R5 are blue and green, respectively. 12% of unpulsed cells are in R2 and 2% in R5. After exposure to 50, 20 ns, 3 MV/m pulses, only 0.1% of the cells remain in the blue R2 region while the green R5 ratio increases to 32%, indicating a change in the accessibility of AT-rich DNA

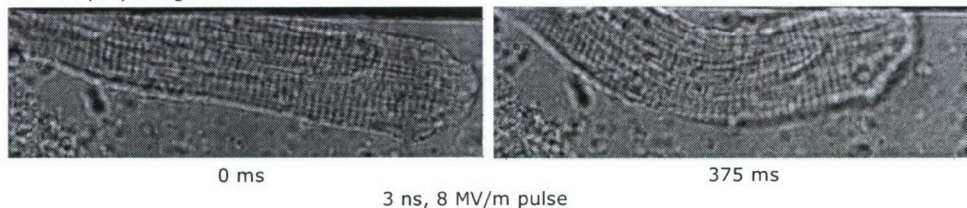
Biological responses. A wide variety of cellular reactions to pulses with widths from 30 ns down to 3 ns have been characterized to a limited extent, including membrane permeabilization and phosphatidylserine externalization [17,31,40], intracellular calcium release [1], chromatin changes (Figure 3), and diagnostic indicators of apoptosis like caspase activation, PARP cleavage, nuclear condensation, and loss of mitochondrial membrane potential [37]. These phenomena have been observed in our laboratories in living cells during pulse exposures in microfabricated electrode chambers [39] and in electroporation cuvettes. Some of the outlines of dose-response curves and the kinetics of the responses have been obtained, but many combinations of pulse parameters, exposure conditions, and cell types remain to be investigated. Experiments performed at Cedars-Sinai Medical Institute in collaboration with clinical oncologists concentrated on cell killing after pulse exposures in electroporation cuvettes and on the responses of tumor implants in nude athymic mice. A wide range of sensitivities among cell types was observed, and we are encouraged by the clinically complete responses of pancreatic tumor implants in mice [11].

The nanoelectropulse responses of the two types of electrically active cells we have studied — cardiomyocytes and adrenal chromaffin cells — are very different, as one might expect, from those of the tumor cells (Figure 4). A single nanosecond pulse causes depolarization, calcium waves, and contraction of cardiomyocyte fibers [6]. Adrenal chromaffin cells also respond to a single pulse as short as 3 ns with elevated intracellular calcium levels and catecholamine release. Preliminary data from experiments with calcium channel blockers indicates that L-type calcium channels participate in this response [41].

ACCOMPLISHMENTS/NEW FINDINGS

Molecular dynamics. MD simulations of phospholipid bilayers in supraphysiological electric fields show a tight association between PS externalization and membrane pore formation on a nanosecond time scale that is consistent with experimental evidence for electropermeabilization and anode-directed PS translocation after nanosecond electric pulse exposure, suggesting a molecular mechanism for nanoelectroporation and nanosecond PS externalization: electrophoretic migration of the negatively charged PS head group along the surface of nanometer-diameter electropores initiated by field-driven alignment of water dipoles at the membrane interface [31].

Cardiomyocyte regulation



Calcium channel gating in adrenal chromaffin cells

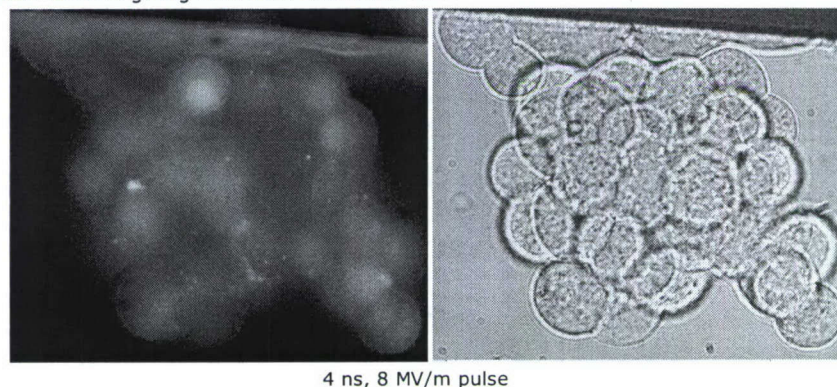


Figure 4. Top. Contraction of rabbit cardiomyocyte after single 3 ns, 8 MV/m pulse delivered to electrodes external to cell. Bottom. Fluorescence image of Calcium Green-loaded bovine adrenal chromaffin cells showing intracellular calcium concentration increase immediately after exposure of the cells to a single, 4 ns, 8 MV/m pulsed electric field.

Pore formation in these simulations of homogeneous lipid bilayers is a molecular process, an extension of water defects into hydrophobic and then hydrophilic, nanometer-diameter pores which takes place in a few nanoseconds. The occurrence of electroporation even in octane membranes suggests that the headgroups play a secondary role. Mechanical forces on the membrane (flexure, tension, compression) are not required for poration in these simulations, beyond the electrostatic torques, repulsions, and attractions expressed at the molecular level, nor are large statistical fluctuations involving the entire transmembrane region required for the nanometer scale electroporation observed in our systems. Water defect propagation into the bilayer interior is enhanced by an energetically unstable alignment of interfacial water dipoles in the applied electric field, which lowers the barrier for this pore-propagating configuration. The extent and direction of the water dipole ordering is influenced by the length and degree of saturation of the lipid hydrocarbon tails and is relatively insensitive to the head group dipole moment and charge. Once a hydrophilic pore has formed, however, the head group dipoles readily rotate and align in the relatively large electric field component tangential to the plane of the pore wall (Figure 5), and this may be important for

Carbon nanotubes. The potential for using carbon nanotubes (CNTs) or other nanowire or nanotube structures as electrodes or sensors in bioelectronics applications has led us to investigate the incorporation of CNTs into our experimental systems. Preliminary unpublished work in our laboratory with SKOV-3 (ATCC HTB-77) human ovarian cancer cells in the presence of single-walled CNTs (SWCNTs) suspended in the medium indicates that these cells endocytotically take up CNTs with no evidence of any immediately toxic effects (Figures 6, 7), consistent with other studies [42,43,44,45,46,47,48].

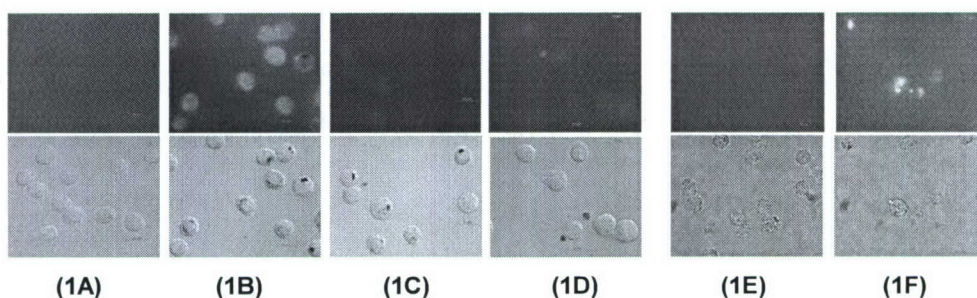


Figure 6. Fluorescein-conjugated CNT (CNT-F) uptake by SKOV-3 cells. (A) Control. (B) Cells incubated with CNT-F, 15 hours. (C) Cells incubated with free CNTs and fluorescein, 15 hours. (D) Cells incubated with free fluorescein, 15 hours. (E) Cells incubated with CNT-F at 4 °C, 1 hour. (F) Cells incubated with CNT-F at 37 °C, 1 hour.

These and other preliminary observations from our work confirm the compatibility of carboxylated CNTs with living cells and suggest that CNTs and other nanostructures may be expected to form intimate contacts with cell membranes and other biomolecular structures (Figure 8).

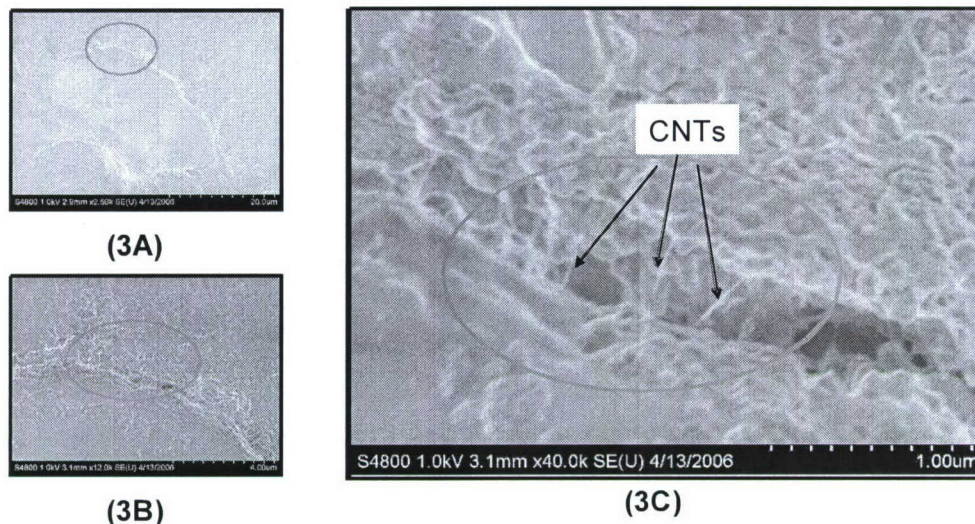


Figure 8. Single-walled CNT interaction with cell membrane. (A) Scanning electron micrograph of SKOV-3 cell. (B) Magnified view of membrane region selected in (A). (C) Higher

Semiconductor nanocrystals (quantum dots). Semiconductor nanocrystal interactions with biological cells have also been investigated in our previous work, directed toward the potential utilization of these quantum dots as reporters (sensors) of nanoelectropulse-induced responses, and possibly as mediators or potentiators of the pulsed electrical field or its effects. We have shown that a quantum dots with a novel core-shell-shell architecture are taken up by some cell types, and that they may be useful as indicators of intracellular pH [49,50].

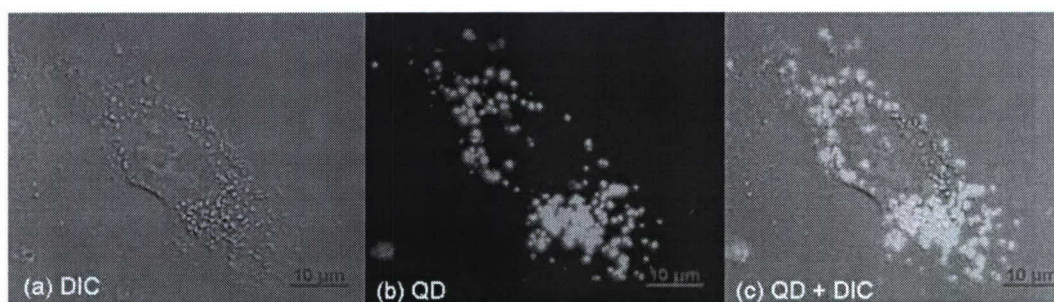


Figure 9. Quantum dot clusters in an SKOV-3 human ovarian cancer cell.

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PERSONNEL SUPPORTED

Martin Gundersen	Professor
Dr. Andras Kuthi	Research Scientist
P. Thomas Vernier	Research Associate Professor
Dr. Yinghua Sun	Graduate Research Assistant (completed PhD, currently postdoctoral research associate at UC Davis)
Dr. Yu-San (Angela) Liu	Graduate Research Assistant (completed PhD, currently research scientist and National Renewable Energy Laboratory
Mr. Meng-Tse Chen	Graduate Research Assistant
Ms. Pavitra Krishnaswamy	Undergraduate, currently enrolled at MIT in EE

PUBLICATIONS

Journal Papers

Possibly the most important paper published during this period is: Garon, E. B., D. Sawcer, P. T. Vernier, T. Tang, Y. Sun, L. Marcu, M. A. Gundersen, and H. P. Koeffler, In vitro and in vivo evaluation and a case report of intense nanosecond pulsed electric field as a local therapy for human malignancies, *Int. J. Cancer* 121:675-682, 2007.

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INTERACTIONS/TRANSITIONS

A. PARTICIPATION/PRESENTATIONS AT MEETINGS, CONFERENCES, SEMINARS, ETC.

Vernier, P. T., M. J. Ziegler, Y. Sun, M. A. Gundersen, and D. P. Tieleman, Nanosecond biomolecular surgery with microelectronics, microfluidics, and nanotubes — in vitro and in silico, Nanomaterials for Defense Applications Symposium, San Diego, 2007.

Vernier, P. T., Y. Sun, M. T. Chen, S. Y. C. Chong, and M. A. Gundersen, DNA-binding fluorochrome photoluminescence in nanoelectropulsed living cells, Bioelectromagnetics Society 28th Annual Meeting, Cancun, 2006.

Vernier, P. T., M. J. Ziegler, Y. Sun, and M. A. Gundersen, Nanosecond electroporation and electrophoretic phosphatidylserine translocation — in vitro and in silico, American Society for Cell Biology 46th Annual Meeting, San Diego, 2006.

Vernier, P. T., Y. Sun, L. Marcu, and M. A. Gundersen, Nanoelectropulse-driven phosphatidylserine externalization and small molecule permeabilization, Biophysical Society Annual Meeting, Long Beach, 2005.

Vernier, P. T. and M. A. Gundersen, Nanosecond, megawatt, millijoule pulses selectively perturb but do not porate mammalian cells, Air Force Office of Scientific Research, Chemistry and Life Sciences Directorate, Bio-Inspired Concepts Review, Annapolis, MD, 2003.

Garon, E. B., P. T. Vernier, J. Wang, T. Tang, M. M. Thu, X. Gu, Y. Sun, L. Marcu, M. Gundersen, H. P. Koeffler, Nanoelectropulse therapy for cancer: in vitro and in vivo analysis, 97th American Association for Cancer Research Annual Meeting, Washington, DC, 2006.

M. Gundersen, "Applications for Compact Portable Pulsed Power: Rocket Science, Cancer Therapy, and the Movies", Proc. 27th International Power Modulator Symposium and 2006 High Voltage Workshop, 2006 (Invited).

Sun, Y., P. T. Vernier, Y.-S. Liu, T. Black, C.-H. Liang, S. Y. Chong, M.-T. Chen, T. Tang, L. Marcu, and M. A. Gundersen, Biophotonic studies of mammalian cells with nanosecond pulsed power using quantum dots, to appear, Proc. 27th International Power Modulator Symposium and 2006 High Voltage Workshop, 2006.

Hao Chen, Chunqi Jiang, Andras Kuthi, and M.A. Gundersen, "High Voltage, Small Back- Lighted Thyratrons", to appear, Proc. 27th International Power Modulator Symposium and 2006 High Voltage Workshop, 2006.

P. Hutcheson, C. Brophy, J. Sinibaldi, C. Cathey, and M. A. Gundersen, "Investigation of Flow Field Properties on Detonation Initiation," 42nd AIAA/ASME/SAE/ASEE Joint Propulsion Conference 2006, Sacramento, California, 9 -12 July 2006.

Hao Chen, Chunqi Jiang, Andras Kuthi, and M.A. Gundersen, "Small Size Back Lighted Thyratrons", Presented to 1st EAPPC 2006, Sep 18-22, 2006 Chengdu, China.

Liu, Y.-S., C. H. Liang, P. T. Vernier, Y. Sun, and M. A. Gundersen, "Design and Synthesis of a Multifunctional Probe for Bio-imaging and Therapeutics" Mater. Res. Soc. Symp. Proc. Vol. 943E, Paper Number 0943-Y03-03, 0943-Y3-03, San Francisco, CA, 2006.

B. CONSULTATIVE AND ADVISORY FUNCTIONS TO OTHER LABORATORIES AND AGENCIES

Visiting Prof. in the Physics Department at the Naval Postgraduate School (NPS).

C. TRANSITIONS. DESCRIBE CASES WHERE KNOWLEDGE RESULTING FROM YOUR EFFORT IS USED, IN A TECHNOLOGY APPLICATION.

The nanopulse technology is now under development by the Alfred Mann Institute (AMI) at USC for applications to skin cancer. The AMI is investing over \$1M to transfer this technology to the medical community for clinical applications. The goal of this development is to produce a practical technology for skin cancer treatment, including basal cell carcinoma.